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(54) Title: ACTIVATION OF T-CELLS BY MODIFIED, ANTIGENIC IMMUNOGLOBULINS

(57) Abstract

A modified immunoglobulin molecule incorporates, preferably in one or more non-CDR loops, one or more foreign antigenic peptides such as a ras peptide. The antigen binding site of the immunoglobulin preferably recognises dendritic antigen presenting cells (APCs). The modified Ig can thus be taken up by dendritic APCs and the foreign antigenic peptide presented on MHC II to naive T-helper cells which stimulate cytotoxic T-cells via the production inter alia of IL-2. Modified Igs of the invention can be used to stimulate the immune system which has apparently become tolerant of a mutant protein, e.g. in the case of certain types of cancer, or it could be used for vaccination against viral infections. The modified Ig can be expressed from recombinant host cells from which it is secreted, notwithstanding the presence of the foreign peptide in a loop of the molecule.

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Activati n of T cells by modified, antigenic immunoglobulins

Field of the invention

This invention relates to the activation of T-cells, and particluarly for stimulating the body's cell-mediated immune system to deal with infection or cancerous mutations in cells.

Background

One of the mechanisms by which the body's immune system operates involves the engulfment of foreign protein by antigen presenting cells (APCs), where the proteins are broken down by proteases into peptide fragments, associated with the cell's MHC II protein in a peptide loading compartment, and then transported to the surface of the APC, where the peptide is presented in association with the MHC II to the T-cell receptors (TCRs) of T-helper cells ($CD4^+$ T-cells). The TCRs of CD4 T-cells only recognise the antigenic peptides in association with MHC II, and the TCRs have a repertoire of recognition sites, so that only those helper cells with the appropriate TCRs will recognise a given antigenic peptide. This activates the helper cell to stimulate cytotoxic T-cells ($CD8^+$ or Tc cells) and B-cells with the corresponding antigen specificity, which then mount an attack on the original source of foreign protein, either directly by $CD4^+$ T-cells themselves, or by the production of antibody (B-cells) or by the Tc-cells. In the case of the latter, receptors on the Tc-cells recognise the original antigen presented in association with MHC I on the surface of cells in which they are endogenously produced, for example as a result of infection or through the generation of a mutated protein within the cell.

Vaccination has been of great importance in the protection against infectious diseases. Even so there is still a need to develop more safe and more effective vaccines. It is also necessary to develop vaccines for diseases for which there until now have been no such preventive measures. For effective vaccination, one needs to activate T-cells of the CD4 type because $CD4^+$ T-cells direct activation of cytotoxic T-cells and B-cells.

Many cancer cells are the result of mutation, for example mutation in the p21 ras gene, and in consequence, an antigenic peptide fragment bearing the mutation is presented at the surface of the cell. The body's immune system may normally deal with such potential cancer cells by receptors on CD4+ T-cells recognising the apparently "foreign" antigen on MHC II and subsequently providing "signal 2" for specific cytotoxic T-cells which may kill the tumour cells. In this way, we probably fight off potential cancers by treating the cancer cells as though they had been infected with a foreign organism. However, in some cases tumours are established, and then it is beneficial to alter the balance between the T-cells and the tumour cells in favour of the T-cells by inducing or increasing specific T-cell activity.

The antigen peptide which locates in the MHC II molecule is typically about 11-20 amino acids in length, and one way of trying to efficiently vaccinate against infectious agents or to activate T-cells specific for tumour specific antigens, is to provide the peptide artificially. However, it has not proved very successful, probably because the peptide is easily degraded.

One group of workers (Zaghouani et al, Science (1993), 259:224-227, and Brumeanu et al, J Exp Med (1993), 178:1795-1799) disclosed the substitution of DNA encoding a viral epitope peptide into the DNA encoding the CDR3 loop of the heavy chain of an immunoglobulin molecule. This gene was co-expressed with a light chain to produce a complete Ig type of molecule, which was taken up by the Fc receptor (FcR) of an APC, and the viral epitope was presented with MHC II on the surface of the APC in vitro. The authors suggested that antigenized self Ig molecules could represent an effective carrier for delivery of peptides to MHC II molecules, as in vaccination or (tolerization) protocols, and a carrier that has a potential to be long-lived and devoid of side-effects.

We have studied antigen presentation of the 91-101 amino acid fragment from the λ2 light chain of M315 antibody (Bogen et al, Eur J Immunol (1986), 16:1373). We have further used in vitro mutagenesis to move the epitope to loops in the human IgG3 heavy chain. Three different mutants were made, each with one of the

loops of the CH1 domain replaced with the 91-101 peptide. The mutant genes were transfected into a fibroblast cell line which had previously been transfected with genes encoding the $E_\alpha^k E_\beta^d$ MHC II. The resulting clones were assayed for the ability to stimulate $\lambda 2^{315}$ specific T-cell clones. It appears that the mutated heavy chains are retained intracellularly in the transfected fibroblasts, but nevertheless the peptide is processed and presented on MHC II.

Summary of the invention

We have found that altered antibodies can be made in which a peptide antigen can be incorporated into a non-CDR loop of an antibody (Ab), and the resulting Ab can be taken up in an APC so that the peptide antigen is presented on the surface of the APC in the context of MHC II, and thereby produce an immune response.

The invention may be used in vaccination against infectious diseases, in which case the peptide antigen, inserted into Ig molecules, should be derived from proteins of the infectious agent. For vaccination purposes, the modified Ig will be targeted to dendritic cells which are especially potent at stimulating naive T-cells.

The invention can also be used in connection with certain types of therapy, the object being to stimulate the immune system which has apparently become tolerant of a particular antigen. For example, many cancers derived from mutations in normal cell proteins may arise because the body's immune system is no longer activated by the mutant protein. Furthermore, the cancer cells are often poor APCs because of a lack of co-stimulatory molecules. A strategy to deal with that, therefore, is to stimulate the body's immune system by presenting it with the mutant protein. Preferably the antigen should be presented on dendritic cells, which are especially potent at stimulating T-cells.

Another aspect of the approach is that peptide antigens presented with MHC II on the surface of APCs stimulate either or both of two types of T-helper cells, Th1 and Th2 respectively. Th1 stimulation results in the production of the cytokines IFN-gamma

and IL-2 and the stimulation of cytotoxic T-cells (Tc). Th2 stimulation, on the other hand, results in the production of the cytokines IL-4, IL-5 and IL-10, leading to activation of B-lymphocytes and the production of antibodies to the antigen. Of the two, the Th1 route is preferred for the present approach, since Tc cells are strong antiviral agents. Also, Tc cells ($CD8^+$ T-cells) with specificity for mutant ras peptide have been detected in cancer patients, and such cancer cells should be susceptible to an enhanced Tc immune response.

The preference for the Th1 or Th2 route depends on the dosage and nature of the antigen administered. A stable antigen will have a longer half-life. As Ig molecules seem to be stable inside an APC, its peptides will be produced at a slow rate at the surface of the APC, giving a low steady state concentration of epitope at the surface, but the antigen will be expected to persist in the APC for a long time. Moreover, an Ig molecule is stable in the organism itself, and therefore the APC may be exposed to the antibody for a long time. Thus, the overall effect of our approach should be a long-lasting low level exposure of the immune system to the antigen carried by the Ig, which may be important for eliciting potent T-cell responses.

We have found that immunoglobulin (Ig) molecules, when engulfed by dendritic cells, stimulate specific naive T-cells to IL-2 production. Moreover, we have found that a modified Ig incorporating an antigen peptide can do likewise.

We have demonstrated that a peptide from a CDR loop of an Ig light chain can be transferred to a loop on the heavy chain constant region, and the Ig will still be expressed.

It is crucial that the main outline of the constant domain framework is maintained after introduction of the peptide, since folding, assembly, retention and degradation are tightly coupled events in the endoplasmic reticulum. Since the peptide loop normally facilitates the correct folding together of the adjacent beta-strands, it may be thought of as being important to allow secretion of the correctly folded molecule when transferred to another loop position.

We have, however, also demonstrated that a ras peptide can be placed on a (non-CDR) loop of an Ig and the Ig still be secreted. This is more surprising, since there is stringent "quality control" in the cells which prevent the Ig from being secreted unless it is properly folded, and altering the amino acid sequence of the loop might be thought to cause the protein to fold into a structure which the cell would detect as incorrect, and hence degrade it.

Having thus demonstrated that important foreign peptides can be introduced into non-CDR loops of an Ig, this can be used to stimulate the immune system, and in particular the Tc cell mediated arm, to attack cells expressing proteins containing such a peptide, especially cancer cells and virally infected cells.

To obtain specific fragments on class II molecules we wish to target these peptide fragments to APCs, more particularly to dendritic cells. This can be done by constructing antibody molecules that have variable regions that will bind membrane proteins on dendritic cells, and which in their constant regions have added foreign antigenic peptide fragments. Such modified antibodies will accumulate on the dendritic cells, be endocytosed, and partially degraded. This process will liberate antigenic peptide fragments that can bind to class II molecules. Peptide fragments/class II molecule complexes will then be transported out to the cell membrane for activation ("vaccination") of $CD4^+$ T-cells.

The present invention is therefore concerned with directing an antigenic peptide to the peptide loading compartment of a dendritic cell APC so that it can be efficiently presented at the cell surface with the MHC II molecule. The approach to this is to insert or substitute DNA encoding the antigenic peptide into the gene of an immunoglobulin (Ig) chain at a position corresponding to a loop of an Ig molecule other than its CDR loops, and especially the loops found in the constant region of the Ig molecule.

A polypeptide chain of an Ig molecule folds into a series of parallel beta strands linked by loops. In the variable region, three of the loops constitute the "complementarity determining

regions" (CDRs) which determine the epitope binding specificity of the antibody. The loops in the constant region do not have antigen binding specificity, but might have a significant effect on the folding of the Ig molecule and/or its effector or other function.

In our invention, the antigen peptide encoding DNA can be substituted for existing non-CDR loop DNA or can be inserted into it. The resulting altered polypeptide chain can become part of a complete Ig type molecule or a fragment thereof such as Fab by combining it with the other chain of the molecule.

The size of the inserted antigenic peptide is probably not critical, so long as it is sufficient to combine with the MHC II of the APC. Generally an 11 amino acid peptide will suffice, but it could be longer, for example 17 or 20 amino acids.

An antigenic peptide could be inserted into just one non-CDR loop, but it may be desirable to incorporate an antigenic peptide into more than one such loop. This could be the same peptide, so as to increase the amount of antigenic peptide that is carried into the APC; or different antigenic peptides could be incorporated, for example to stimulate a patient's immune system simultaneously with respect to more than one antigen, or to provide on a single molecule antigenic peptides characteristic of more than one strain of an infectious organism or antigenic peptides from the same antigen binding to different MHC II. The loading capacity of the Ig molecule refers to the number of non-CDR loops which may have antigen peptides incorporated into them.

Thus, references to an immunoglobulin or Ig molecule will, unless the context indicates otherwise, be generally construed to include complete Ig-type molecules with intact heavy and light chains, apart from the modifications to incorporate the peptide antigens, fragments of such complete molecules, and single chain versions of the molecule, whether by omitting a heavy or light chain or by covalently linking the chains end-to-end, as well as other variations in the normal Ig molecule as may be desired. The Ig may be of any class or subclass (IgG_1, IgG_2, IgG_3, IgG_4, IgA, IgE, IgM etc).

The non-CDR loops utilised may be in the CH1 or C_x region between the hinge region and the variable region. This would apply particularly where the Ig is a Fab fragment. Alternatively or additionally, loops in the Fc region can be us d.

A preferred feature of the present invention is that the antigen-binding specificity of the variable region of the Ig molecule is kept unaltered. In this way, the Ig carrying the antigen peptide can be targeted to an appropriate site on the APC by selecting an antibody specific for the relevant surface molecule on the APC. For example, the variable region of the antibody may be specific for an MHC molecule or a marker for dendritic cells, or for an FcR molecule. In the latter case, the modified Ig would bind the FcR by recognition of the variable region, rather than (or possibly in addition to) recognition of the Fc region of the Ig molecule. Entry of the modified Ig into the APC in this way could be regarded as "head first" as distinct from the "sideways" entry in the case of Fc recognition. Sideways entry via FCγI does not necessarily require retention of antigen specificity in the variable region, but there are advantages in having the variable regions with retained antigen specificity, even in that case. A particularly preferred approach is to use antigen binding regions which are for dendritic cell markers. Such a marker is CD11c and antibodies to this marker are also known (N416, ATCC HB224).

Thus, such antibodies, or their variable regions, can be used in the kind of modified antibody constructs described herein. If cross-linking of the antibody on the surface of the dendritic cells is required, this can be provided by employing bispecific antibodies of the present invention, so that one of the variable regions can be specific to a cross-linking entity, or by employing also an antibody against (eg the Fc region of) the altered antibody of the present invention. On the other hand, if the variable region of the Ig is not be specific for an APC surface epitope, but rather the Fc region of the altered antibody is used to target the FcR on the APC, the variable region can then be used to bind to a cross-linking entity, the antigen thereby forming an immune complex which is readily taken up via the FcR.

In general, therefore, the advantages of retained antigen-binding specificity for the APC (preferably a dendritic cell) include the following.

It provides potentially greater binding affinity to APCs than Fc-FcR binding.

It provides potentially divalent binding, which can increase the likelihood of being taken up by and degraded within the APC.

It provides the possibility of the Ig molecules being cross-linked by their constant regions, thereby further increasing the likelihood of being taken up by the APC.

It provides the possibility (in the case of bispecific antibodies) of the Ig molecules being cross-linked by their variable domains, thereby increasing the likelihood of being taken up and correctly processed by the APC, since FcRs are primarily adapted to take up immune complexes.

It can substantially prevent the Ig from being targeted to the wrong site.

It allows one in principle to target any of the surface epitopes of the APC, and in particular epitopes specific to dendritic cells.

The present invention therefore provides, inter alia:

modified Ig molecules having antigenic peptides in one or more non-CDR loops;

the utilisation of such modified Ig molecules to provide a medicament for immunisation or therapy in a patient, especially where the patient suffers, or is at risk of suffering, from an infection or cellular mutation involving this antigenic peptide.

DNA encoding such Ig molecules, and the expression of that DNA in a suitable host cell to provide the modified Ig;

Production of modified Igs of the present invention

The methods for producing antibodies and antibody fragments by recombinant DNA expression are now well known and utilised. Those methods can be similarly used in carrying out the present invention. Typically, for the production of Fab fragment molecules of the present invention, the V genes are fused with C_K (light) and CH1 (heavy) genes in the same vector, and by the same known methods as for production of complete antibodies.

Detailed description

The recombinant DNA methods are further illustrated by the following examples.

In the accompanying drawings:

Fig 1 shows:

(A) a restriction map of the human Cγ3 gene. Exons are shown as boxes. h=HindIII, bg=BglII, p=PstI, s=SphI, b=BamHI. PstI and BamHI sites outside the 2.6kb gene construct reside in the pUC polylinker.

(B) amino acid sequence of the human IgG3 C_H1 domain. For each mutagenesis reaction one of the segments was replaced with the 91-101 ($\lambda 2^{315}$) epitope (lower line).

(C) nucleotide sequences of the primers used for the in vitro mutagenesis reactions. The inserted nucleotides encoding the 91-101 epitope are in the central section, with 21 flanking nucleotides on either side.

Fig 2 shows two expression vectors using the human CMV promoter to express inserted antibody gene sequences:

(A) pLNOH2 for expression of any heavy V-gene in combination with any C-gene;

(B) pLNOK for expression of any kappa V-gene in combination with a kappa C-gene.

(C) pLNOH2/pLNOK layout.

Fig 3 is a graph showing IL-2 produced after stimulation of lymph node cells with antigenic peptide using the L3 mutant Ab or the native M315 Ab as antigen.

Fig 4 shows:

(A) The γ3 constant region as cloned in pUC19. Exons are shown as boxes. H=HindIII, Bg=BglII, P=Psti, S=Sphi, B=BamHI. PstI and BamHI sites outside the 2.6kb gene construct reside in the pUC polylinker.

(B) Amino acid sequence (single letter code) of the human IgG3 C_H1 domain. The four amino acids constituting the CDR3-corresponding loop in C_H1 are shown in bold. These were replaced with amino acids encoding the 1-25 ras or 5-21 ras epitope.

(C) Nucleotide sequences of the primers used for the *in vitro* mutagenesis. The inserted nucleotides encoding the 1-25 or 5-21 ras epitope are in bold and the 20 nucleotide flanking regions are in non bold.

Fig 5 shows the nomenclature and constructs of constant region genes for IgG3 with different ras epitopes. Exons are shown as boxes. Amino acids constituting the CDR3-corresponding loop are given as single letter aa symbols. Underlined aa are changed relative to the ras w.t. sequence.

<u>The construction and expression of mutant IgG3 heavy chains carrying a CDR3 loop peptide insert</u>

Fig 1 shows the insertion of DNA encoding the 91-101 ($\lambda 2^{315}$) antigenic peptide from the CDR3 region of the M315 Ig myeloma protein at three different positions in the C_H1 domain of the human γ3 chain polypeptide. Using conventional in vitro mutagenesis, the inserted DNA encoding the 11 amino acid peptide epitope replaced respectively seven, seven and four amino acids in the L1, L2 and L3 loops of C_H1, the new positions of the epitope being chosen such that they were similar for all mutants with respect to secondary structure.

The resulting C_H1 encoding fragment is used to replace the wild-type C_H1 domain of a complete Cγ3 gene using the HindIII and BglII sites, and the Cγ3 gene can be subcloned as a HindIII-BamHI fragment into an expression vector.

Referring to Fig 2; these expression vectors are intended for expression of Igs from PCR-amplified V-genes together with a genomic C-gene construct. The vectors have the following features.

Allows expression of intact V-genes. Whole, intact V-genes are amplified using PCR-primers within the leader region and the constant region respectively.

Allows easy subcloning of V-genes. Non-abundant restriction sites flanking the V-genes are introduced in a PCR-reamplification reaction. Computer analysis of V-genes have been done to exclude restriction sites frequently found within the V-gene coding region.

Allows expression of Ig with any C-gene. The vectors have been constructed with CHγ3 and C_κ constant region genes, and these genes may be exchanged with other constant gene sequences.

Contains hCMV-promotor. The hCMV-promotor gives high expression in several mammalian cell lines.

Neomycin selection marker for stable expression.

SV40 ori for transient expression. Transient expression gives the possibility for fast analysis of the gene product.

Allows isolation of single stranded vector DNA. f1-origin of replication within the vector gives the possibility for isolation of single stranded DNA for direct sequencing and in vitro mutagenesis of the immunoglobulin genes.

No co-transfection of heavy and light chain vectors. A single cloning step makes the combination vector: pLNOH2K. This is an easy way to avoid cotransfection of the two vectors.

In the vector pLNOH2, upstream from the cloning site the vector contains a hCMV promoter and also the murine V_H gene, V_{NP}, thus

creating a complete chimeric heavy chain gene. The V_{NP} gene segment codes for a V_H chain characteristic of a λ1 light chain-bearing mouse antibody with specificity for the hapten 4 hydroxy-3 nitrophenacetyl (NP) and the iodinated derivative 5 iodo-4 hydroxy-3 nitrophenacetyl (NIP). The pLNOH2 vector also contains a neomycin selection marker that allows selection of stably transfected cells by virtue of their resistance to G-418.

Fab fragments can be produced employing an expression vector (pLNOH2/Fd) which is a modification of pLNOH2, and is made as follows. PCR primers were constructed which hybridized in the 5' intron and in the very 3' end of the CH1 exon of the human Cγ3 gene. The primers were constructed with restriction enzyme tags such that the sense primer

5'-ACGTACGCTAGCTTTCTGGGGCAGGCCAGGCCT-3'

introduced a BsiWI site (underlined), and the non-sense primer

5'-ATCGGATCCTCAAACTCTCTTGTCCACCTTGGTG-3'

introduced a BamHI site (underlined) as well as a stop codon (bold). The PCR product was inserted in pLNOH2 on BsiWI and BamH1 sites to substitute the Cγ3 gene and make pLNOH2/Fd. In pLONH2/Fd the Ig transcript is polyadenylated by the BGH pA site in the vector.

PCR amplification and reamplification of the V-genes

The human or mouse heavy and light(kappa) V-gene are amplified with appropriate primers (Larrick et al, BioTechnology, Vol 7, 1989, 934-938; Bendig et al, BioTechnology, Vol 9, 1991, 88-89) within the leader and the constant region if the complete V-genes are to be preserved.

In order to clone the PCR-amplified V-genes into the vector, the V-genes are sequenced and reamplified with primers which hybridize with the V-gene. The primers should also include tags that incorporate (see Fig 2):

for the 5' primer: a part of the leader sequence with restriction site BsmI

for the 3' primer: splice/donor site and restriction site HpaI or BsiWI.

Cloning of the C-genes

The C genes in the vector are exchanged with C genes carrying the antigenic peptides, which are produced as described in Figure 1.

The vectors have incorporated a human constant γ3 gene (for pLNOH2) and a human constant kappa gene (for pLNOK) on HindIII/BamHI restriction sites. These H/B fragments include introns with branch point signal and splice acceptor signal as well as a poly-A signal in the 3' end. C-genes cloned into the H/B restriction site should include these signals.

Primers for reamplification

When constructing reamplification primers for a VH-gene the splice donor site aggtgagt should be included (see above).

When constructing primers for a VK-gene the splice donor site tgagtagaa from human kappa J1 segment can be used.

3' VK primer 22147:
5' cgtacgttctactc*acgtttgatctccagct* 3'
BsiWI splice VK

5' VK primer 22146:
5' ggtgtgcattcc*gacattgagctcacc* 3'
BsmI VK

3' VH primer VHTP3F:
5' cgtacgactcacct*gaggagacggtgac* 3'
BsiWI splice VH

5' VH primer VHTP3B:
5' ggtgtgcattccc*aggtccaactgcag* 3'
BsmI VH

The sequences given in italics are examples of V-sequences. The primers constructed should in each case be complementary to the

actual V-genes that are to be amplified.

Combination of the two vectors in one

pLNOK is cut with BgIII and BamHI. This makes a fragment containing the hCMV promotor, V-gene and the Kappa gene. The fragment can be inserted into the pLNOH2 vector at a BamHI site in either direction.

The above two vectors can be used in non-B cells, such as fibroblast host cells, to express the Ig chains under the hCMV promoter, which gives high level expression. If a plasma host cell is used, these vectors with the hCMV promoter can also be employed, although the natural Ig promoter would function, albeit at a lower level of expression.

Expression of the mutant heavy chain in J558L cells using an Ig promoter

The mutant C3 genes were subcloned as Hind III - BamHI fragments (see Fig 1) into the vector pSV2gptV_{NP} [Neuberger et al. 1985, Nature 314:268-271]. These constructs were transfected into the J558L cell line. $2x10^7$ cells and 20 g DNA in 800 l PBS was transfected using electroporation conditions of 3.5 kV/cm and 25 F. The J558L cell line is a plasmacytoma cell line which is no longer producing its heavy chain. When transfected with heavy chain genes, however, complete Ab can be produced and secreted.

Ab containing L3 mutant heavy chains (see Fig 1) were isolated from the growth medium of the transfected cells by use of two successive columns coated with Protein A and Protein G, respectively. The Protein A column was used to remove some of the contaminating Ab from the FCS, but will not bind human IgG3. The mutant L3 Ab was eluted from the Protein G column with 0.1 M glycine-HCl, pH 2.7.

Isolation of spleen cells enriched for dendritic cells

Spleens were injected with 0.5 ml 100U/ml collagenase type IV and incubated for 5 min, and then the spleen cells were isolated. The red blood cells were lysed in ACT and the remaining cells

incubated in petridishes at 37°C for 2 h. Nonadherent cells were removed, fresh medium added and the dishes were incubated over night at 37°C. After swirling of the dishes, nonadherent cells were collected and used for the T cell activation experiments.

T cell activation assays for exogenously added antigenic L3 Ab

Lymph node (LN) cells from T cell receptor transgenic mice [Bogen et al, Eur J Immunol (1992), 22:703-709] were used as T cells, and as APC, BALB/c spleen cells enriched for dendritic cells were employed. LN cells (10^5/well) and irradiated (2000 rad) spleen cells (10^4/well) were cocultured in triplicates with various amounts of antigenic L3 Ab. Two days later, the cultures were pulsed for 24 h with 1μCi [^{3}H]dThd, and [^{3}H]dThd incorporation counted. Just prior to pulsing of the cells, samples of the supernatant was removed. Their IL-2 content was measured by the use of the IL-2 dependent CTLL cell line, as previously described [Lauritzen, G.F. and Bogen, B. 1991, Scand. J. Immunol. 33, 647-656].

Results from a T cell assay with exogenously added antigenic L3 Ab

We wanted to see if the antigenic peptide could be excised from the L3 mutant heavy chain and presented to T cells also when added exogenously to APC. APC (spleen cells) and T cells (LN cells) were cocultured in triplicates in medium containing various amounts of L3 Ab. A positive control using 10 μg/ml synthetic 91-101($\lambda 2^{315}$) peptide as antigen was also included. T cell proliferation was measured both by incorporation of [^{3}H]dThd, and by IL-2 secretion into the culture supernatant. The results from the IL-2 measurements are given in Fig 3. The most IL-2 (incorporation of [^{3}H]dThd into CTLL cells, cpm) was measured in the controls using synthetic peptide as antigen (not shown). The average cpm from these controls was 42,276, approximately three times as much as with 10μg/ml L3 antigen. With increasing amounts of antigenic M315 or L3 Ab, however, significant levels of IL-2 was secreted into the culture supernatant. Notably, it seems that the peptide is more efficiently presented when located in the L3 mutant than when in its original position in M315. Very similar results were

obtained when measuring T cell proliferation.

The construction of an altered IgG3 heavy chain carrying various mutant ras peptide inserts

Immunological reagents

The antibodies and conjugates used for ELISA were made in our laboratory. Biotinylation was performed as described [Goding J. W. (1986) Monoclonal Antibodies: Principles and Practice. *Sc.ed. Academic Press, London*]. Biotin-X-NHS was obtained from Calbiochem Corporation (La Jolla, CA). The hapten NIP/NP labeling of BSA or Sepharose has been described [Michaelsen T. E., Aase A., Westby C. and Sandlie I. (1990) Enhancement of complement activation and cytolysis of human IgG3 by deletion of hinge exons. *Scand J Immunol* 32, 517-528; and Sandlie I., Aase A., Westby C. and Michaelsen T. E. (1989) C1q binding to chimeric monoclonal IgG3 antibodies consisting of mouse variable regions and human constant regions with shortened hinge containing 15-47 amino acids. *Eur J Immunol* 19, 1599-1603.]. NIP and NP/NIP-Cap-o-Su were purchased from Cambridge Research Biochemicals Ltd., Cambridge, UK.

In vitro mutagenesis and construction of mutant IgG3 heavy chain genes

(1) Construction of genes encoding IgG3 1-25ras and IgG3 5-21ras

The human γ3 constant region gene (coding for the G3m(b∘) allotype) is contained on a 2.6kb HindIII-SphI fragment cloned into the polylinker of pUC19 (a gift from M.-P. Lefranc, Laboratoire d' Immunogénétique, Université des Sciences et Techniques du Languedoc, Montpellier, France) (pUCγ, Figure 4A). A 0.9 kb HindIII-PstI fragment encoding the C_H1 domain was subcloned into the polylinker of M13mp18. *In vitro* mutagenesis was performed as described by Kunkel and others [Kunkel T. A., Roberts J. D. and Zakour R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth Enzymol* 154, 367-382; and Sanger F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463-5467.]. To ensure efficient annealing in spite

of the large heteroduplex generated, we chose to have flanking regions of 20 nucleotides on each side of the mutagenic core nucleotides. Figure 4B and C show the amino acids changed and the primers used.

Reagents and *E.coli* strains used for mutagenesis were purchased from BIO RAD laboratories (Richmond, CA, USA) and synthetic oligonucleotides from the DNA Synthesis Laboratory, University of Oslo or from MedProbe AS (Oslo, Norway). Screening for mutants was facilitated by the introduction of a unique SpeI restriction site. The mutations were verified by sequencing employing the Sanger dideoxy chain termination method (5). Reagents used were supplied in the Sequenase 2.0 commercial kit from United States Biochemicals (Cleveland, Ohio, USA).

(2) Construction of genes encoding IgG3 1-25ras and IgG3 5-21ras with single amino acid substitutions in the ras epitope

To introduce single amino acid substitutions in the ras epitope in IgG3 1-25 ras and IgG3 5-21 ras, the C_H1 domain from these gene constructs was cloned into M13mp18. These constructs served as templates for the *in vitro* mutagenesis reactions. The mutations introduced were as follows:

The primer 5'-tgggcgcggtgggcgtgggc-3' was used to make the 12Gly→Val mutation (IgG3 1-25rasG12V and IgG3 5-21rasG12V), the primer 5'- gtgggcgcgcggggcgtgggc-3' was used to make the 12Gly→Arg mutation (IgG3 1-25rasG12R and IgG3 5-21rasG12R), and the primer 5'-gcgcgggcgacgtgggcaagt-3' was used to make the 13Gly→Asp mutation (IgG3 1-25rasG13D and IgG3 5-21rasG13D). Nucleotides in bold mark the sites of mutations. Figure 5 depicts the resulting mutant IgG3 heavy chain genes.

The mutated C_H1 fragments were substituted for corresponding wild type (w.t.) sequences on HindIII-BglII sites in pUCγ. The mutant IgG3 heavy chain genes, as well as the w.t. gene, were cloned as HindIII-BamHI fragments into the vector pLNOH2 (see Fig 2). Upstream from the cloning site, this vector contains a hCMV promoter and also the murine V_H gene, V_{NP}, thus creating a complete chimeric heavy chain gene. The V_{NP} gene segment codes for a V_H chain characteristic of a λ1 light chain-bearing mouse

antibody with specificity for the hapten 4 hydroxy-3 nitrophenacetyl (NP) and the iodinated derivative 5 iodo-4 hydroxy-3 nitrophenacetyl (NIP). The pLNOH2 vector also contains a neomycin selection marker that allows selection of stably transfected cells by virtue of their resistance to G-418.

Cell culture and gene transfer

The mutant heavy chain genes were introduced into the murine myeloma cell lines J558L (a gift from Dr. S.L. Morrison, Dept. of Microbiology, Molecular Biology Institute, UCLA) or NS0 (obtained from ATCC) by electroporation. J558L produces a λ1 light chain but expresses no heavy chain of its own. The Vλ of the endogenous light chain complements the V_{NP} of the transfected heavy chain to yield an NP-specific antibody. NS0 does not make any immunoglobulin polypeptide at all, and was therefore transfected with a combination vector made of pLNOH2 containing the IgG3 heavy chain genes and the vector pLNOK (see Fig 2) containing a human κ light chain gene inserted downstream of an irrelevant mouse variable gene. Thus, the antibodies secreted from NS0 do not bind the haptens NP/NIP.

J558L cells were maintained in RPMI 1640 and NS0 in DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco BRC, Paisley, Scotland) at 37°C and 5%CO_2.

Transfections were carried out by subjecting $2x10^7$ cells and 20 μg plasmid in 0.8 ml ice-cold PBS to an electric field of 3.5 kV/cm using a capacitance setting of 25 μF. Cells were then diluted in regular medium and seeded in 24 well tissue culture dishes. After incubation for 24 hours, transfectants were selected in medium supplemented with 800 μg/ml G-418 (Gibco). Colonies of stably transfected cells were visible after approximately 2 weeks. The transfection efficiency was about $5x10^{-5}$.

Quantification of chimeric antibodies

The amount of IgG3 secreted by transfectants was measured by ELISA. Microtitre plates were coated overnight at 4°C with 1

μg/ml BSA-NIP or 2 μg/ml polyclonal sheep anti-human IgG (Fab specific) in PBS/0.02% azide. After washing several times with PBS containing 0.05% Tween 20 (PBS/T), 100 μl cell supernatant were added to each well and incubated at 37°C for 1.5h. After washing as above, a second layer was added, consisting of biotin-labeled polyclonal sheep anti-human IgG (γ chain specific) (1:8000) together with streptavidin and biotin-labeled alkaline phosphatase (1:6000) in PBS/T. After an additional 1.5h incubation at 37°C and repeated washing with PBS/T, bound antibody was revealed by addition of the substrate p-nitrophenylphosphate (Sigma). The reaction was carried out for 20-60 min at 37°C before absorbance at 405 nm was measured on a Dynatech MR 700 Microplate Reader. Standard curves of antibodies were constructed by measuring the absorbance of serial dilutions of affinity purified human IgG that previously had been quantitated by ELISA and by measuring absorbance at 280 nm.

Secretion of mutant antibodies from J558L and NS0 cells

J558L was transfected with the heavy chain gene constructs described in Figure 5. NS0 was transfected with the IgG3 w.t., IgG3 1-25ras and IgG3 5-21ras heavy chain gene constructs together with the κ light chain gene. The amount of antibodies secreted was determined by an ELISA reaction where the hapten antigen (NIP-BSA) was used as coat. Since normal levels of antibody were detected, this shows that the antigen specificity is retained after introduction of the peptide in CH1.

The amount of antibodies secreted by J558L transfectants is summarized in Table 1.

The amount of antibodies secreted from NS0 transfectants does not differ significantly from the J558L transfectants (see Table 2). However, we have recently obtained individual clones of NS0 transfectants which secrete 5-10 μg/ml of IgG3 1-25ras and IgG3 5-21ras antibodies (data not shown).

Table 1. Amount of antibodies secreted by J558L transfectants

Antibody produced	Secretion level (μg/ml)[1)]	Fraction of stably transfected cells secreting antibodies (%)[2)]
IgG3 w.t.	0.90 ± 0.70	95
IgG3 1-25ras	0.40 ± 0.60	50
IgG3 1-25ras G12V	0.65 ± 0.50	61
IgG3 1-25ras G12R	0.60 ± 0.60	57
IgG3 1-25ras G13D	0.65 ± 0.65	83
IgG3 5-21ras	0.60 ± 0.50	70
IgG3 5-21ras G12V	0.70 ± 0.60	58
IgG3 5-21ras G13D	1.10 ± 1.00	48

[1)] Secretion level for the different transfectants as determined by ELISA (described in *Materials and methods*). Measurements were made from 20-50 individual colonies of each of the J558L transfectants.
[2)] The fraction of individual colonies secreting antibodies as determined by ELISA.

Table 2. Amount of antibodies secreted by NS0 transfectants

Antibody produced	Secretion level (μg/ml)[1)]	Fraction of stably transfected cells secreting antibodies (%)[2)]
IgG3 w.t.	1.50 ± 1.30	100
IgG3 1-25ras	0.90 ± 0.60	35
IgG3 5-21ras	1.40 ± 1.40	67

[1)] Secretion level for the different transfectants as determined by ELISA (described in *Materials and methods*). Measurements were made from 20-40 individual colonies of each of the NS0 transfectants.

[2)] The fraction of individual colonies secreting antibodies as determined by ELISA.

CLAIMS:

1. A modified immunoglobulin molecule or a functional fragment or part thereof (herein referred to as a modified Ig), having an antigenic peptide foreign to the Ig incorporated in one or more non-CDR loops.

2. A modified Ig of claim 1, wherein the antigen binding domain of the Ig recognises a cell surface molecule of an antigen presenting cell (APC).

3. A modified immunoglobulin or a functional fragment or part thereof (herein referred to as a modified Ig), having antigenic peptide foreign to the Ig incorporated in its structure, and a recognition site for dendritic antigen-presenting cells (herein referred to as dendritic APCs).

4. A modified Ig according to claim 3 wherein the recognition site for dendritic APCs is provided by the antigen binding site of the Ig.

5. A modified Ig according to claim 3 or claim 4 wherein the foreign antigenic peptide is incorporated into a loop of the protein molecule.

6. A modified Ig according to claim 5 wherein the foreign antigenic peptide is incorporated into a non-CDR loop of the molecule.

7. A modified Ig of any one of the preceding claims, wherein antigenic peptide is incorporated into one or more loops of the constant region of the heavy or light chain.

8. A modified Ig of any one of the preceding claims, wherein the antigenic peptide represents a tumorigenic mutation in a cellular protein.

9. A modified Ig of any one of claims 1 to 7 wherein the antigenic peptide represents an epitope of a virus or other infective organism.

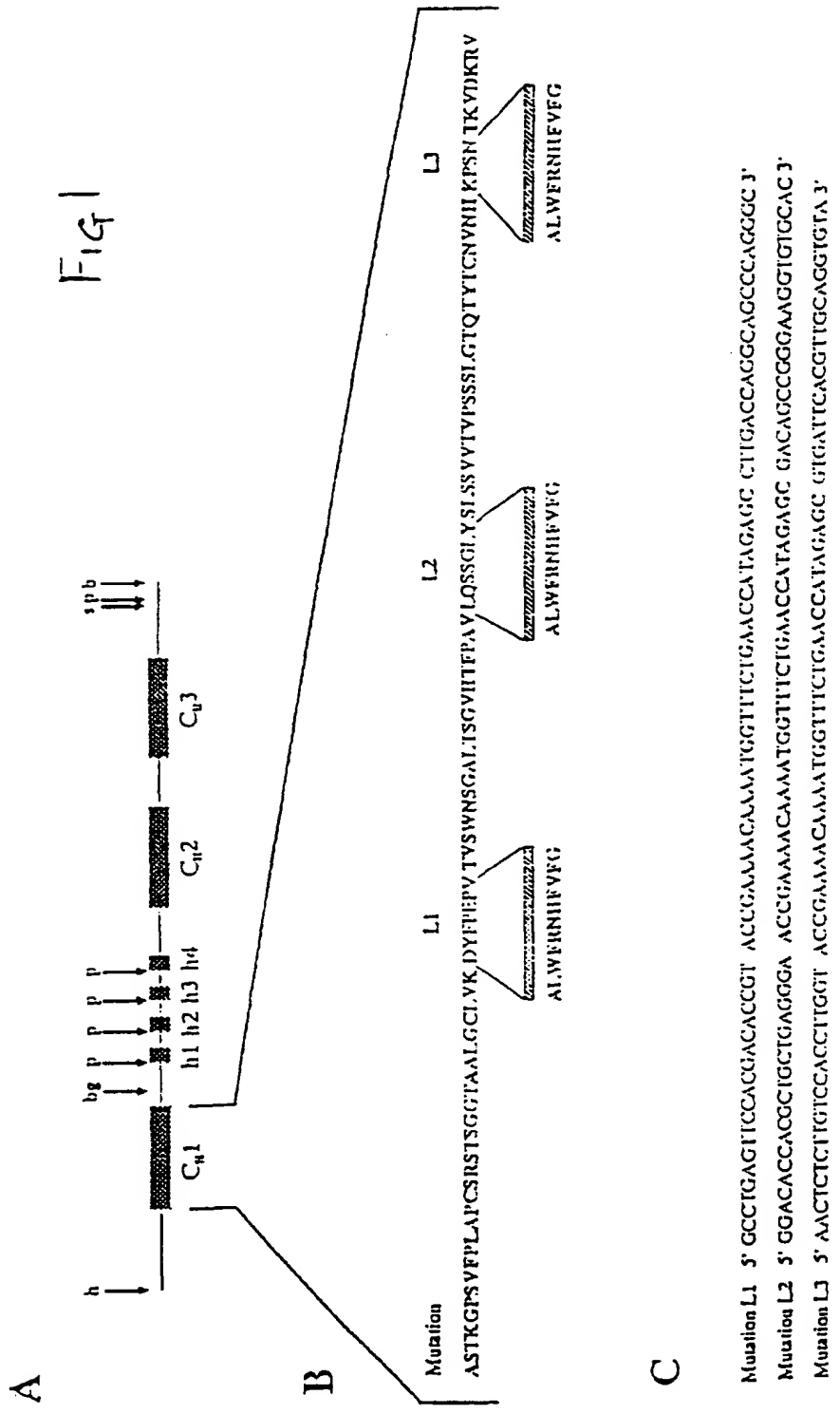
10. A modified Ig of any one of the preceding claims for therapeutic or prophylactic use.

11. The use of a modified Ig molecule of any one of claims 1 to 9 in the preparation of a medicament for immunisation or therapy, where the patient suffers, or is at risk of suffering, from an infection or cellular mutation involving an epitope represented by said antigenic peptide.

12. DNA encoding a modified Ig of any one of claims 1 to 9.

13. The expression of DNA of claim 12 in a suitable host organism to produce said modified Ig.

FIG 1


A
h
bg
p
p
p
p
h1 h2 h3 h4
Cγ1
Cγ2
Cγ3
spb
B
Mutation
ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRV
L1
L2
L3
ALWFRNHFVFG
ALWFRNHFVFG
ALWFRNHFVFG
C
Mutation L1 5' GCCTGAGTTCCACGACACCGT ACCGAAAACAAAATGGTTTCTTGAACCATAGAGC CTTGACCAGGCAGCCCAGGGC 3'
Mutation L2 5' GGACACCAGGCTGCTGAGGGA ACCGAAAACAAAATGGTTTCTTGAACCATAGAGC GACAGCCGGGAAGGTGTGCAC 3'
Mutation L3 5' AACTCTCTTGTCCACCTTGGT ACCGAAAACAAAATGGTTTCTTGAACCATAGAGC GTCATTCACGTTGCAGGTGTA 3'

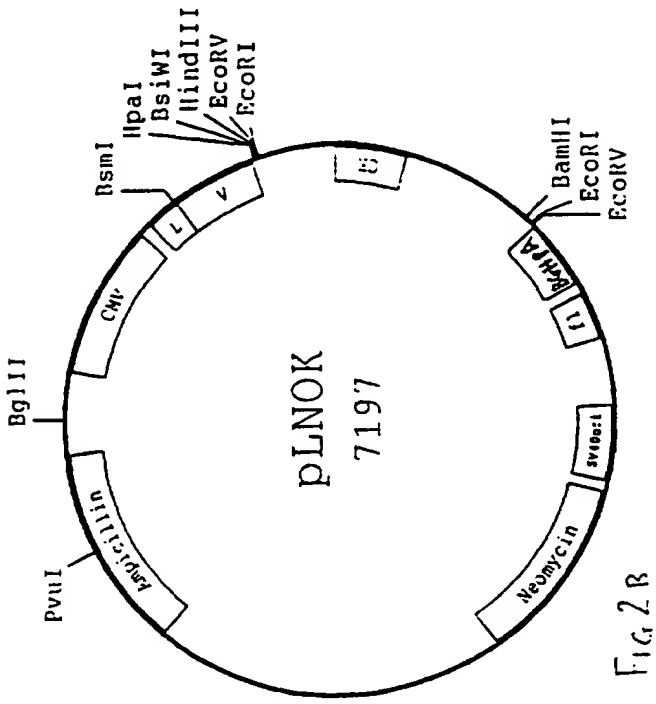


Fig 2B

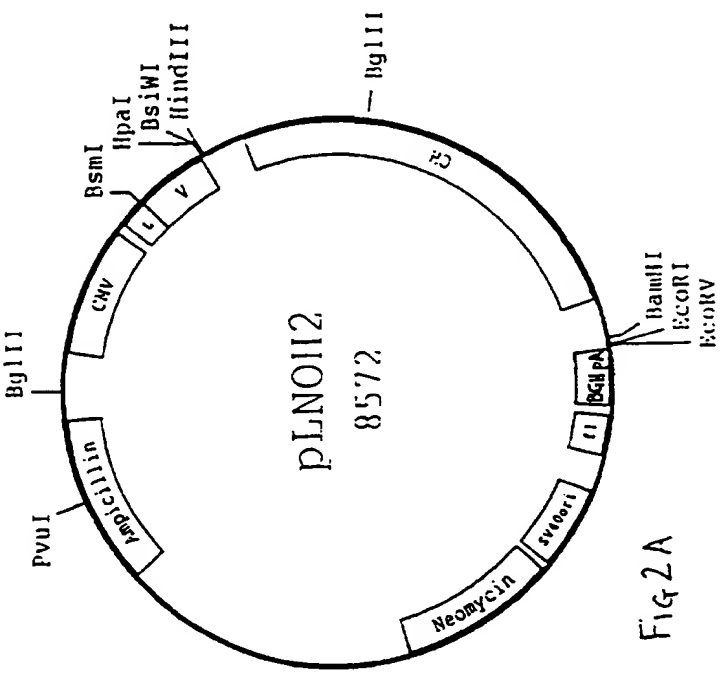


Fig 2A

Fig 2C

leader V-gene intron C-gene

1 2 3 4 5 120

G V H S Q V Q L Q T V S S

5'..gtgtgcattccaggtccaactgcag.....acagtctcctcaggtgagtttacgtacgcgtacgc.....xxxxxxxxxxxxxxxxxx.....ggatcc 3'

BsmI Splice donor HpaI BsiWI HindIII Splice acceptor BamHI

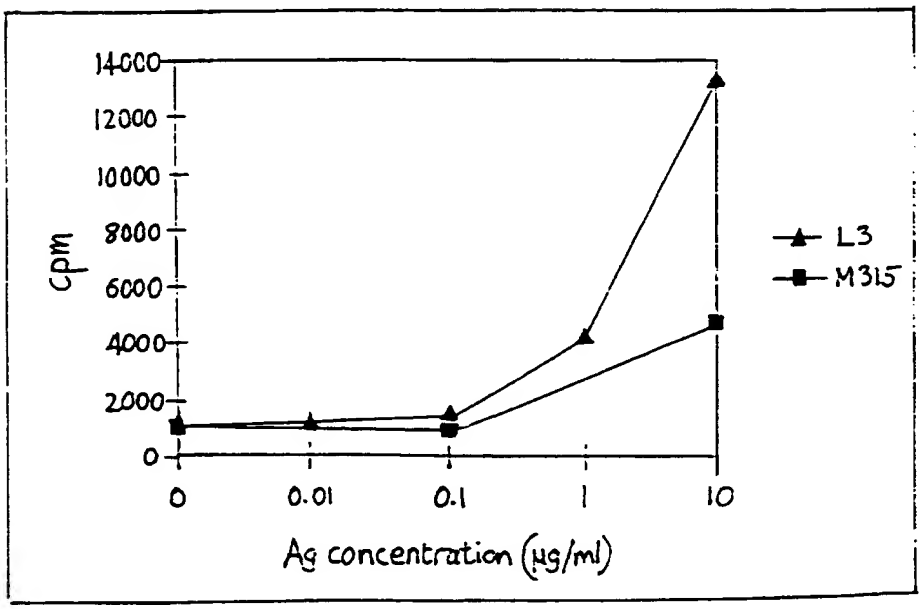

14000
12000
10000
8000
6000
4000
2000
0
cpm
0
0.01
0.1
1
10
Ag concentration (µg/ml)
L3
M315

Fig 3

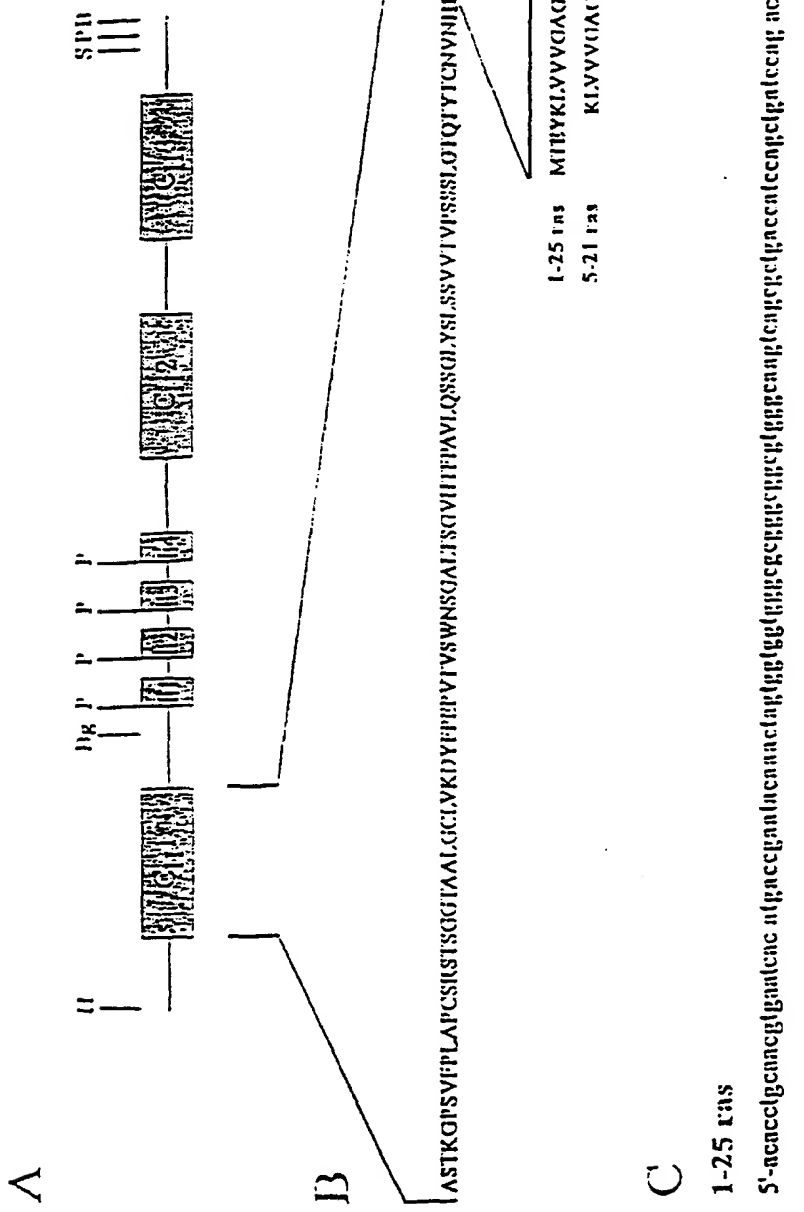
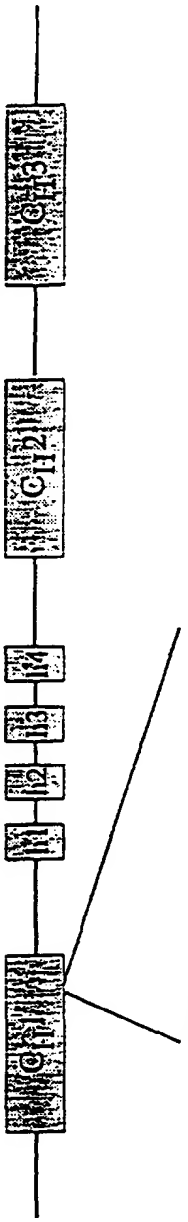

A
B
ASTKDPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
1-25 ras MTEYKLVVVGAGGVGKSALTIQLIQ
5-21 ras KLVVVGAGGVGKSALTI
C
1-25 ras
5'-acaccatgactgaatataaacttgtggtagttggagctggtggcgtaggcaagagtgccttgacgatacagctaattcag acaaatggtgaacaagagagt-3'
5-21 ras
5'-acaccatgactgaatataaacttgtggtagttggagctggtggcgtaggcaagagtgccttgacgatacagctaattcag acaaatggtgaacaagagagt-3'

Fig 1



KSPN

IgG3 w.t. MTPYKLVVVGAGGVGKSALTIQLIQ

IgG3 1-25 ras MTPYKLVVVGAGGVGKSALTIQLIQ

IgG3 1-25 ras G12V MTPYKLVVVGAVGVGKSALTIQLIQ

IgG3 1-25 ras G12R MTPYKLVVVGARGVGKSALTIQLIQ

IgG3 1-25 ras G13D MTPYKLVVVGADGVGKSALTIQLIQ

IgG3 5-21 ras KLVVVGAGGVGKSALTI

IgG3 5-21 ras G12V KLVVVGAVGVGKSALTI

IgG3 5-21 ras G12R KLVVVGARGVGKSALTI

IgG3 5-21 ras G13D KLVVVGADGVGKSALTI

Fig 5

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/GB 96/00116

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 09393 (IGEN INCORPORATED) 5 October 1989 see examples see claims	1,7,10, 12,13
A	WO,A,94 14847 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 7 July 1994 see examples see claims	1,10-13
A	WO,A,94 14848 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 7 July 1994 see examples see claims	1,10-13

-/--

[X] Further documents are listed in the continuation of box C.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 1996	23. 04. 96

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/00116

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 10, October 1994 WEINHEIM, GERMANY, pages 2542-2547, XP 000567079 O. BREKKE ET AL. 'Human IgG isotype-specific amino acid residues affecting complement-mediated cell lysis and phagocytosis.' see abstract ---	1,7,10, 12,13
A	CLINICAL AND EXPERIMENTAL IMMUNOLOGY, vol. 97, no. 3, September 1994 OXFORD, GB, pages 361-366, XP 000567067 S. PEIFANG ET AL. 'Enhanced activation of human T cell clones specific for virus-like particles expressing the HIV V3 loop in the presence of HIV V3 loop-specific polyclonal antibodies.' see the whole document ---	1,9-11
A	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, November 1993 NEW YORK, NY, USA, pages 1795-1799, XP 000567066 T. BRUMEANU ET AL. 'Efficient loading of identical viral peptide onto class II molecules by antigenized immunoglobulin and influenza virus.' cited in the application see the whole document ---	1,9-11
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 86, no. 1, January 1989 WASHINGTON, DC, USA, pages 282-286, S. WEISS ET AL. 'B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells.' see abstract ---	1-13
P,X	WO,A,95 31483 (ECLAGEN LIMITED) 23 November 1995 see the whole document -----	1-13

1

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INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/GB 96/00116

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8909393	05-10-89	AU-B-	3342689	16-10-89
		CA-A-	1335656	23-05-95
		IL-A-	89690	08-12-95
WO-A-9414847	07-07-94	NONE		
WO-A-9414848	07-07-94	NONE		
WO-A-9531483	23-11-95	AU-B-	2452195	05-12-95

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Published
With international search report.

(54) Title: COMPOSITIONS AND METHODS FOR ENHANCING IMMUNE RESPONSES MEDIATED BY ANTIGEN-PRESENTING CELLS

(57) Abstract

Molecular adjuvants are disclosed comprising an antigen presenting cell-targeting ligand functionally linked to an immunogen, e.g. tumor associated antigens, bacterial or viral antigens, etc. Methods are disclosed for delivery of these molecular adjuvants to patients, resulting in the transduction of activating signals to the targeted antigen presenting cell, thereby enhancing the immune response to the co-delivered immunogen.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16825

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 424/185.1, 192.1, 277.1; 530/324, 326, 328, 395, 403
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/185.1, 192.1, 277.1; 530/324, 326, 328, 395, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN-BIOSCIENCE cluster, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ---- Y, P	DEMPSEY, P.W. et al. C3d of Complement as a Molecular Adjuvant: Bridging Innate and Acquired Immunity. Science. 19 January 1996, Vol. 271, pages 348-350, see entire document.	1-3 ---- 7, 10-11, 13, 17-21, 24
A	EP 0 145 174 A1 (SUNTORY KABUSHIKI KAISHA) 19 June 1985 (19.06.85), see entire document.	1-3, 8-10, 11, 13, 17, 18-24
A	SANDERSON, S.D. et al. Decapeptide Agonists of Human C5a: The Relationship between Conformation and Neutrophil Response. J. Med. Chem. 1995, Vol. 38, pages 3669-3675, see entire document.	3-7, 16-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
15 JANUARY 1997	24 JAN 1997

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INTERNATIONAL SEARCH REPORT

Inter[illegible]ional application No.
PCT/US96/16825

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/08, 38/10, 38/17, 38/21, 38/30, 39/385, 39/39; C07K 1/04, 7/06, 7/08, 14/52, 14/705, 14/74, 17/00

The approaches described above have met with varying degrees of success in stimulating the immunogenicity of weakly antigenic or non-antigenic substances. However, they provide only a generalized stimulation of immunity, and are not designed to target specific populations of immune system cells (such as antigen presenting cells). A desired objective in effecting therapeutic internvention in various disease states is to provide a means for specifically targeting a protein or peptide to a population of antigen-presenting cells and thereby stimulate those cells to internalize the antigen of interest and present it to the immune system in an effective, specific context. Insofar as it is known, such a system is not currently available.

SUMMARY OF THE INVENTION

The present invention provides novel compositions and methods for delivering specific antigens to antigen-presenting cells, and simultaneously delivering signals to those cells that produce a desired immune response. The compositions of the invention are sometimes referred to herein as "APC-targeted activating antigens."

According to one aspect of the invention, these APC-targeted activating antigens, which elicit an immune response mediated by an antigen-presenting cell, comprise at least one antigenic moiety functionally linked to at least one targeting moiety that binds specifically to a characteristic determinant on the antigen-presenting cell. For purposes of the present invention, the term "functionally linked" is defined generally as linking of the moieties in such a way that each moiety retains its intended function. This is particularly relevant with respect to the targeting moiety, which is designed to bind to a characteristic determinant on the antigen-presenting cell.

Antigen-presenting cells contemplated for

antibody titer in the sera of mice collected either before (pre) or after immunization with peptides 3 (YKQGGFLGLYSFKPMPLaR) and 4 (YSFKPMPLaRKQGGFLGL) as determined by radioimmunoassay and shows the relationship between the amount of ^{125}I-goat anti-mouse antibody bound and the dilution factor of mouse sera which had been incubated in microtiter wells coated with MUC1 epitope peptide. Note that peptides 3 and 4 comprise two moieties, a targeting ligand and an antigen to which an immune response is desired.

Figure 3 is a graph illustrating the titers of antibody classes and subclasses produced in mice following immunization with peptide 3 (YKQGGFLGLYSFKPMPLaR) as determined by ELISA using rabbit anti-mouse IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM, followed by goat anti-rabbit conjugated to peroxidase and detected using p-nitrophenyl phosphate cleavage monitored at 405 nm.

Figure 4 is a graph illustrating the specificity of binding of the antibody subclasses in sera from mice immunized with peptide 3 (YKQGGFLGLYSFKPMPLaR) as determined by ELISA using binding to microtiter wells coated with MUC1 epitope peptide and detection with rabbit anti-mouse IgG2a, IgG2b, or IgM followed by incubation with goat anti-rabbit conjugated to peroxidase and detected using p-nitrophenyl phosphate cleavage monitored at 405 nm.

DETAILED DESCRIPTION OF THE INVENTION

A major obstacle in the development of vaccines and other immunostimulatory agents is the inability of some antigens to be readily taken up and processed by antigen presenting cells. Uptake of antigens by APCs is an essential step for stimulating an effective immune response, since the immune system recognizes the antigen

utilized. Such analogs are described in detail in commonly-owned U.S. Application Serial No. 08/299,285, the entire disclosure of which is incorporated by reference herein.

An exemplary C5a C-terminal decapeptide agonist preferred for use in the present invention is:

YSFKPMPLaR

This decapeptide is a potent agonist of naturally occurring C5a, and is preferred to naturally occurring C5a because its small size contributes to ease of synthesis and solubility. Moreover, these conformationally biased peptides are stable toward serum carboxypeptidase digestion, express a level biological selectivity, and have been shown to be non-toxic in high concentrations in athymic mice.

Peptide analogs of naturally-occurring interferon γ are also contemplated for use in the present invention. Peptides corresponding to the amino terminal 39 amino acids of IFNγ have been shown to compete for binding with native IFNγ. Antibodies against this domain block biological activity, and removal of the first 10 amino terminal residues eliminates biological activity. This suggests that binding of IFNγ to its cognate receptor is mediated by this portion of the molecule. Accordingly, peptides based on this domain are contemplated to be of use for delivering antigens to APCs expressing IFNγ receptors. In this regard, it should be noted that human and mouse IFNγ are absolutely species specific in binding and activity. Consequently, for stimulating APC-mediated immune responses in mice, the mouse peptides will be utilized, and the human peptide will likewise be utilized for stimulating APC-mediated immune responses in humans. The mouse IFNγ 39 amino acid peptide analog is composed of the following sequence:

HGTVIESLESLNNYFNFFGIDVEEKSLFLDIWRNWQKDG

The human IFNγ 39 amino acid peptide analog is composed of the following sequence:

QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEE

Another ligand cont mplated for use in the present invention is the C3dG component of complement. This component is a 348 residue fragment derived by proteolytic cleavage from the C3b precursor (residue 955-1303 of C3; Swissprot accession p01024). C3dG can be converted to C3d (residues 1002-1303) and C3g (residues 955-1001). C3dG and C3d remain associated with non-activator surfaces and serve as opsonins for phagocytosis by macrophages and other antigen presenting cells. Cd 21 is the C3dG and C3d receptor.

The above-listed ligands exemplify the type of ligand preferred for practice of the present invention. However, it will be appreciated by those skilled in the art that other ligands may be utilized as the targeting moiety of the APC-targeted antigens of the invention. These include ligands that are already known in the art, as well as ligands that may be discovered and developed henceforth. Antibodies or antibody fragments also may be used to target APC-specific cell surface antigens.

The type of antigen that can be chosen as the antigenic moiety in the present invention can be any peptide, polypeptide or derivative thereof for which an immune response or antibody production is desired. These include but are not limited to, peptides, polypeptides (i.e. proteins) and derivatives thereof, such as glycopeptides, phosphopeptides and the like. Synthetic peptide and polypeptide derivatives or analogs, or any other similar compound that can be conjugated to a receptor-targeting moiety can be used in the present invention. Moreover, these peptides, proteins and derivatives may comprise single epitopes or multiple epitopes for generating different types of immune responses. Indeed, if an entire protein is conjugated to a targeting moiety, this protein is likely to comprise numerous epitopes, which may vary d pending upon the solvent conditions and their effect on secondary and

most well-characterized tumor associated epitopes described to date for MUC1 are found in the tandem repeat domain. These include carbohydrate structures and protein structures. MUC1 tumor associated epitopes are well characterized, and thus have been proposed to be used for the production of tumor vaccines using conventional methods. Exemplary compositions of the present invention comprise MUC1 epitopes, such as those set forth below, as the antigenic moiety of the APC-1 targeted antigens of the invention, to demonstrate the potential of the present invention as potent tumor vaccines.

MUC1 epitope predicted to bind to class I molecules of the H-2k^b type has sequence homology to the juxtamembrane region of MUC1;

YKQGGFLGL

MUC1 tandem repeat has the sequence:

GVTSAPDTRRAPGSTAPPAH

The components comprising the APC-targeted antigens of the invention can be made separately, then conjugated. For example, a small peptide analog, such as the above-described C5a agonists, may be produced by peptide synthetic methods, and conjugated to a protein which has been purified from naturally occuring biological sources. Alternatively proteins engineered for expression via recombinant methods may be used. Additionally, targeted antigens comprising peptide components (i.e., a peptide antigenic epitope conjugated to a peptide receptor ligand) can be synthesized in tandem by peptide synthetic chemistry according to known methods and as described in greater detail below. Finally, targeted antigens of the invention comprising two larger polypeptide moieties (i.e., a large polypeptide antigen linked to a large ligand) can be made by recombinant techniques. For example, DNA molecules encoding both components can be ligated together by

ethylcarbodiimide methiodide (EDC). As described in greater detail in Example 2, this method was used to conjugate a C5a C-terminal decapeptide analog to serum amyloid A (SAA). Methods for joining two proteins together are also available.

The peptides or proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, they may be subjected to amino acid sequence analysis, mass spectra analysis or amino acid compositional analysis according to known methods.

E. General Formulae and Exemplary Compositions of the Invention

The APC-targeted antigens of the invention can comprise one or more antigenic moieties, and likewise can comprise one or more targeting moieties. Moreover, these moieties can be functionally linked in several ways. For instance, if "T" represents a targeting moiety, and "Ag" represents an antigenic moiety, the APC-targeted antigens of the present invention may be organized as follows:

Ag - T;

T - Ag;

T_1 - Ag - T_2;

T_1 - $[Ag]_n$ - T_2 (wherein $[Ag]_n$ represents a multiplicity of antigens.

Examples of the general formulas set forth above include:

Ag - C5a agonist peptide;

IFNγ peptide - Ag;

IFNγ peptide - $[Ag]_n$ - C5a agonist peptide.

Other representive compositions of the invention include:

MUC1 Class I binding epitope - C5a agonist C-terminal peptide

Murine or human IFNγ peptide - MUC1 Class I binding epitope

Murine or human IFNγ peptide - MUC1 tandem repeat
MUC1 Class I epitope - -C3dG peptid
SAA-K-Ahx - C5a C-terminal peptide (Ahx = ϵamino hexanoic acid).

It will be appreciated by persons skilled in the art that the APC-targeted activating antigens of the invention may be adapted for inclusion of large or complex antigens. This may be accomplished, for example, by inclusion of a "spacer" (such as the K-Ahx spacer moiety in the exemplary compound above) between the antigen and the targeting moiety. Such chemical modifications are familiar to biochemists.

II. Uses of APC-Targeted Activating Antigens

The APC-targeted activating antigens of the present invention have broad potential for clinical applications in humans and animals. As discussed above, a significant impediment to the development of vaccines and other immunotherapeutic agents is the apparent inability of particular antigens to be readily taken up and processed by antigen presenting cells. The compositions of the invention faciliate the specific delivery of an antigen to a population of antigen presenting cells, whereupon the delivery mechanism (e.g., using as the targeting moiety a receptor ligand capable of transducing a biological signal) simultaneously activates the antigen presenting pathway. of the APC. Thus, the present invention enables development of vaccines and other immunotherapeutics that can specifically target any peptide antigen or other antigenic structure covalently attached to a ligand for a receptor present on an antigen presenting cell. It is believed that antigens linked to ligands that selectivity bind to and activate a particular population of APCs can not only generate an immune response, but can influence the nature of the immune respons that is generated. Thus, immune respons s that favor antibody, cellular, Th1

responses for certain antigens.

As a step toward developing anti-cancer vaccines for clinical use, the compositions of the invention can be used to advantage as research tools to further explore the effect of stimulating a certain population of APCs with a tumor antigen and determining the effect on an anti-tumor immune response. To this end, it should be noted that the present application exemplifies targeted antigens comprising an epitope of a particular tumor-specific antigen, Mucin-1.

Previous tumor vaccine formulations that aim to immunize patients with compounds that are identical to compounds already produced by tumors have proven to be of limited value, probably because tumors that progress have been selected for their lack of immunogenicity in their respective host (e.g., the host is tolerant to existing tumor antigens). Thus, one important challenge of producing effective tumor vaccines is generating reagents that counteract immunological tolerance to tumor-associated antigens. One purpose of the APC-targeted antigens described above is to induce in the immunized individual a response against their tumor that is similar to that seen in individuals undergoing allograft rejection. In other words, the goal is to induce an autoimmune reaction against the tumor that is capable of destroying the tumor. The immunological parameters that regulate tolerance to tumor antigens are not well understood; nonetheless the compositions described herein have the potential to counteract this tolerance and thus induce specific immune responses that mediate tumor rejection.

The targeted antigens of the present invention will also find broad utility in the production of antibodies for use as immunodiagnostic and immunotherapeutic agents. For immundiagnostic purposes, antibodies are wid ly used in various quantitative and qualitative assays for the detection and measurement of

biological molecules associated with diseases or other pathological conditions. For reasons that often are not well understood, it is sometimes difficult to generate antibodies against certain biological molecules using conventional means. The compositions of the present invention provide an alternative means for inducing an animal to produce antibodies against a weakly-antigenic or non-antigenic substances. The utility of the compositions of the invention in this regard is shown clearly in Example 2, below, in connection with serum amyloid A. The appearance and abundance of this protein in the body is strongly correlated with systemic inflammatory stress, which is a condition that is very difficult to quantitate. It is believed that quantitative assays for SAA levels would be an excellent indicator of general, systemic inflammation; thererfore it would be of benefit to generate antibodies against the protein in a non-human species. This protein has proved particularly recalcitrant to the generation of antibodies using conventional measures. As described in Example 2, a targeted antigen comprising SAA conjugated to a C5a peptide ligand produced a significant antibody response in mice injected with the conjugated molecule. In a similar fashion, targeted antigens comprising any weakly-antigenic or non-antigenic component of interest could be made and used to produce specific antibodies in laboratory animals, for use as immunodiagnostic reagents.

Antibodies for use as immunotherapeutic agents can also be generated using the compositions of the invention. As one example, there has been a great deal of recent interest in developing reagents capable of down-regulating or inhibiting the complement cascade to modulate local and systemic inflammatory responses. To this end, the C3a convertase, which is active early in the cascade, could provide an ideal target for complement inhibition. C3a convertase cleaves the peptide C3 into two components, C3a and C3b, and therefore must be able

to access the cleavage site on C3 in order to accomplish the result. Antibodies directed toward the C3a-C3b cleavage site are expected to be effective in blocking access of C3a convertase to the cleavage site, thereby inhibiting this early step in the complement cascade. Such antibodies may be generated using a targeted antigen of the invention comprising, as the antigenic moiety, the short peptide sequence comprising the C3a/C3b cleavage site. The sequence could then be conjugated to an appropriate targeting moiety, such as the C5a C-terminal decapeptide agonists exemplified herein. Thus, the compositions would be useful to generate an immunotherapeutic agent (e.g., an antibody that blocks the activity of C3a convertase) for treating an adverse inflammatory condition.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate the invention in greater detail. They are not intended to limit the invention in any way.

EXAMPLE 1

Evaluation of Mucin Epitope (MUC1/C5a agonist) Conjugate for Recruitment and Activation of Antigen Presenting Cells (APCs) and Stimulation of an Immune Response in Mice

The C5a receptor is present on numerous antigen presenting cells, including monocytes, macrophages, dendritic cells, and other cell types. In this example, a composite peptide comprising a mucin epitope (MUC1) fuctionally linked to a decapetide agonist analog of C5a corresponding to the C-terminal effector region of the natural factor was evaluated for its ability to activate the APCs thereby stimulating an immune response in mice. This evaluation is based on the known property of C5a receptors to internalize and recycle in the antigen pr s nting cell, thereby acting as id al candidates for delivering antigens to and simultaneously activating

signals in the APCs. Because C5a receptors ar particularly common on macrophag s, monocyt s and dendritic cells, it is believed that the use of a C5a agonist analog to bind C5a receptors will result in preferential activation of these APCs.

i. Abbreviations. Except where noted, the single letter designation for the amino acid residues is used: alanine is A; arginine is R; asparagine is N; aspartic acid is D; cystine is C; glutamine is Q; glutamic acid is E; glycine is G; histidine is H; isoleucine is I; leucine is L; lysine is K; methionine is M; phenylalanine is F; proline is P; serine is S; threonine is T; tryptophan is W; tyrosine is Y; and valine is V. Upper case letters represent the L-amino acid isomer and lower case the D-isomer.

ii. Peptide synthesis, Purification and Characterization. The following peptides were synthesized according to standard solid-phase methodologies on an Applied Biosystems (Foster City, CA) model 430 A peptide synthesizer and characterized as previously described (7):

(1) The antigenic "juxta-membrane" (JM) epitope of the human mucin-1 (MUC1), YKQGGFLGL;

(2) The C5a C-terminal decapeptide agonist analog, YSFKPMPLaR;

(3) The composite peptide YKQGGFLGLYSFKPMPLaR, in which the JM epitope is positioned toward the amino terminus and the C5a peptide is positioned toward the carboxyl terminus; and

(4) The composite peptide YSFKPMPLaRKQGGFLGL, in which the JM epitope of MUC1 is positioned toward the carboxyl terminus and the C5a analog is positioned toward the amino terminus.

Peptide 3 retains C5a biological activity, whereas peptide 4 does not because the biologically important carboxyl terminal end of the C5a analog is

Group D. mice immunized with peptide (3)
Group E. mice immunized with peptide (4).

The results of the experimental protocols are set forth in Figures 1 and 2. As can be seen in the Figures, the mice in Groups A, B, C and E produced no appreciable increase in antibody response to inoculation with MUC1 epitope (Group A), C5a agonist peptide (Group B), MUC1 epitope combined with, but not conjugated to, C5a agonist peptide (Group C), or MUC1 epitope conjugated to the C5a agonist peptide at its C-terminus, rather than its N-terminus (thereby blocking C5a biological activity) (Group E). Only mice inoculated with the MUC1 epitope/C5a agonist peptide conjugate of the present invention (Group D) generated an appreciable antibody response. Furthermore, this stimulation was significant. It is clear from these results that inoculation with the conjugated MUC1 epitope/C5a agonist peptide was far more efficient in stimulating a general immune response (i.e, production of antibodies) than was inoculation with either peptide alone, or both peptides together, but not conjugated, or peptides conjugated in the opposite orientation.

There are several significant conclusions that can be drawn based on these results. The fact that both Balb/c and C57B16 mice showed antibody responses to peptide 3 suggests that the antigen presenting effect is not restricted by MHC haplotype. The fact that immune responses were not produced to peptide 4, or to mixtures of peptide 1 and 2, but that substantial responses were produced to peptide 3, suggest that the effect is mediated by the C5a moiety of the peptide and that the immune response results from the simultaneous delivery of antigen peptide and C5a mediated activation signals to antigen presenting cells.

The isotypes of the anti-peptide antibodies produced in the immunized mice were determined (Figure 3)

and were found to consist of IgM, IgG2a, and IgG2b. This sugg sts that the immunogenic peptide is producing T cell-dependent responses, which generally require antigen processing and presentation. Data presented in Figure 4 show that the antibody response to peptide 3 includes a high percentage of antibodies that are specific for the MUC1 epitope that was the antigen moiety of these studies.

EXAMPLE 2

Evaluation of Serum Amyloid A/C5a Peptide Conjugates for Recruitment and Activation of APCs and Stimulation of Immune Response in Rats

Serum amyloid A is an acute-phase stress response protein generated by the liver. Along with other acute phase proteins, SAA is secreted in response to systemic inflammatory stress as a protective measure. SAA is of interest because it appears to be an excellent indicator of general, systemic inflammation, which is a phenomenon that is very difficult to quantitate. Because serum levels of SAA have been observed to parallel the rise and fall of the systemic inflammatory response, quantitation of serum levels of this peptide would provide an effective means of assessing inflammation. One way to accomplish this is to develop antibodies against SAA that could be used for quantitation such as in an ELISA assay. However, SAA has been particularly recalcitrant to the generation of antibodies against it. In this example, an evaluation was made of the ability of SAA conjugated to a C5a C-terminal analog (as described in Example 1) to activate antigen producing cells and produce an antibody response in rats.

i. Production and preparation of proteins and p ptid s. The C-terminal C5a analog K-Ahx-YSFKPMPLaR (AhX is aminohexanoic acid, which is a linear aliphatic

spacer moiety) was produced as described in Example 1. The aliphatic spacer moiety was included to separate the critical receptor-binding C5a analog from the bulky protein to be attached to the amino terminus.

Serum amyloid A was conjugated to the C5a peptide analogs according to the following method. SAA (100 μg) was reacted with a 50-fold molar excess of a water soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodiide (EDC), in 200 μl of phosphate buffered saline, pH 7.5, at room temperature for 30 minutes. A 50-fold molar excess of the peptide (K-Ahx-YSFKPMPLaR) and a 100-fold molar excess of a base diisopropylethyl amine (DIEA) were added to this solution. Water was added to the solution to bring the reaction mixture to a volume of 400 μl. This solution was stirred overnight at room temperature and then lyophilized to a dry powder. The powder was diluted to the appropriate volume with water to generate the stock mixture used for inoculating the animals.

ii. Experimental protocols. Rats were injected intraperitoneally with an inoculant comprising the SAA/C5a peptide conjugates in phosphate-buffered saline with or without RIBI adjuvant. Booster injections were given two and five weeks after the initial injections. The rats were sacrificed seven weeks after the initial injection and anti-mucin antibody production was assessed from the serum titers, as described in Example 1.

Significant anti-SAA antibody was produced from both groups of rats, whether or not RIBI adjuvant was included in the inoculation. As visualized by gel electrophoresis and autoradiography of anti-SAA antibody eluted from the plate assays, it appeared that anti-SAA antibody titers were essentially equivalent, or slightly higher, in rats inoculated with SAA/C5a peptide conjugate in the absence of RIBI adjuvant as compared to the same

inoculation without the adjuvant. Thus, antigenic conjugates comprising the C5a peptide analog are useful for generating antibodies against large proteins, as well as against smaller peptide fragments, such as those described in Example 1. Moreover, the successful generation of anti-SAA antibodies utilizing this method is particularly promising for purposes of producing antibodies against weakly- or non-antigenic peptides or proteins.

EXAMPLE 3

Production and Characterization of Site-Directed Neutralizing Antibodies Specific for a Peptide κR(33-52) from the Predicted Amino-Terminal Region of the Human Kappa Receptor

Receptors for human opioid peptide hormones have been described on numerous cell types. The receptors for μ, κ, and δ ligands have recently been cloned from genomic and cDNA libraries derived from normal tissue and cell lines. Considerable homology exists among the μ, κ, and δ receptors, except for the N-terminal regions of the receptors. The N terminal region of the human kappa receptor (amino acid residues 1-100) is relatively hydrophilic and would be predicted to be exposed on the surface of the cell membrane. A 20 residue peptide [κR(33-52)], was chosen and used to raise a site directed peptide specific polyclonal antibody (5).

The method of production of a polyclonal antiserum in rabbits using the molecular adjuvant, C5a-agonist peptide conjugated to the κR epitope is set forth below. The binding specificity and biological activities of the resulting polycolonal antiserum raised to the predicted extracellular region of the human kappa receptor (κR) are also described below.

i. Construction of Targeted-Immunogen. A peptide construct consisting of the κR(33-52) (FPGWAEPDSNGSAGSEDAQL) covalently attached to the N-terminal end of a conformationally biased, C5a

complement fragment agonist analogue peptide (YSFKPMPLaR) was synthesized according to the methods in Example 1 and as previously reported (7).

ii. Preparation of anti-κR(33-52) Antiserum and Peptide-Specific ELISA. Rabbits were immunized s.c. with 500 μg of FPGWAEPDSNGSAGSEDAQLYSFKPMLaR construct in complete Freund's adjuvant (GIBCO, Grand Island, NY) on day O followed by booster injections on days 30 and 60 in incomplete Freund's adjuvant. Serum was collected starting 75 days after the initial immunization.

The presence of anti-peptide antibody was determined by using a peptide specific ELISA utilizing the free κR(33-52) peptide as previously described (8). Anti-κR(33-52) and normal rabbit γ-globulin (RGG) were purified by protein A Sepharose chromatography (Sigma) (8) prior to use.

iii. Cells and culture conditions. The neuroblastoma cell SK-N-SH (HTB 11), ductal breast cell carcinoma T47D (HTB 133) , Jurkat T cell leukemia, (TIB 152), U937 histolytic lymphoma (CRL1593), THP 1 human monocyte (TIB 202), EBV-transformed B cells SKW 6.4 (TIB 215) and CESS (TIB 190) (American Type Culture Collection, Rockville, MD) were cultured in DMEM or RPMI 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 1 mM L-glutamine, 2 mM Na pyruvate, 50 U penicillin and 50 μg/ml streptomycin. The human neuronal precursor cells NT2 (Stratagene, La Jolla, CA) were cultured in Opti-MEM (Gibco) supplemented as above. All cultures were incubated at 37° C in a humidified chamber with 7.5% CO_2.

Peripheral blood derived mononuclear cells were obtained from healthy male and female volunteers, isolated by Ficoll-Hypaque(tm) density gradient centrifugation and enriched for macrophage by adherence to plastic.

iv. Flow Cytom try. Single-color flow cytometry analysis of cells (1 X 10^6) in PBS containing 1% bovine calf serum and 0.1% sodium azide (staining buffer) were preincubted 30 minutes at 4° C in the presence of 20% normal human serum. The cells were washed and then incubated with anti-κR(33-52) or RGG for 30 minutes at 4° C, washed and labeled with PI-conjugated donkey (Fab')2 fragments of antirabbit IgG (Zymed, S. San Francisco, CA) for 30 minutes at 4° C (8). For dual color analysis FITC-conjugatd anti-CD3 or anti-CD14 (Pharmingen, San Diego, CA) were also included in the second step. Cells (1 x 10^4) were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) and data were analyzed with the Cell Quest software as previously described (8).

v. Measurement of cell proliferation. Peripheral blood mononuclear cell (PBMC) were pulsed on day 2 of culture with ^{3}H-thymidine and 18 hours later the cells harvested on glass fiber filters and processed for scintillation counting. Experiments were performed three times and each sample done in triplicate.

vi. Measurement of IgG Secretion. Relative levels of IgG in culture supernatants were determined by an indirect ELISA as previously described (9). Supernatant derived from PBMC cultures were collected after 10 days and assayed for the presence of IgG. Numbers represent the mean CPM +/- SD from triplicate samples. Experiments were performed at least three times.

vii. Characterization of Anti-κR Peptide Antisenum. Serum from rabbits immunized with the κR(33-52)YSFPMPLaR construct and normal rabbit serum were assayed for the ability to recognize plate bound κR(33-52) in ELISA. The results show that serum from rabbits immunized with the κR(33-52)YSFPMPLaR construct bound free κR(33-52) peptide in a dose dependent fashion. The titer was approximately

10^5. In contrast, serum from unimmunized rabbits failed to bind this peptide. Serum samples from immunized and unimmunized rabbits were subjected to protein A-Sepharose chromatography and the column eluates were assessed for κR(33-52) specific antibody. The results indicate that protein A-purified antibody derived from rabbits immunized with the κR(33-52)YSFPMPLaR construct binding to free κR(33-52) was detectable at antibody concentrations less than 0.1 ng/ml. In contrast, RGG failed to bind the free peptide. The results from multiple bleedings indicated that the ED_{50} titer ranged between 1-10 ng/ml. These results indicate that rabbits immunized with KR(33-52)YSFPMPLaR contained high titer, κR(33-52) peptide specific antibody.

viii. Binding of anti-R (33-52) antibody to cells expressing human κR. To determine whether the polyclonal anti-κR(33-52) antibodies bound to cells expressing the κR, a variety of mononuclear cell lines and normal human mononuclear cells were first assayed for the presence of the κ receptor specific mRNA by RT-PCR. RNA samples isolated from neuronal cell lines NT2, U937, Jurkat, T47D, normal human PBMC, and enriched human macrophage were subjected to RT-PCR analysis with 5' sense and 3' antisense primers specific for the 3' region of the cloned κR and B-actin. All of the cell lines or cell fractions, except for the T47D cell line, were positive for the κ-receptor specific PCR product, as expected based on the primer sequences used (5).

Experiments were performed to determine whether anti-κR(33-52) bound to cells expressing κR specific mRNA. The results of single color flow cytometric analysis for several cell lines are shown in Table 2. Flow cytom tric measurements were conducted with human cell lines representative of macrophage (U937), T

Table 1

Select d cell type binding of anti-κR(33-52) antibodies produc d in rabbits immunized with C5a-agonist peptide conjugated to the κR(33-52) sequence as assessed by single channel color flow cytometric analysis.

		Mean Channel Intensity	
Cell Line	Cell Type	RGG	anti-κR Ab
NT2	Neuronal	9	19
U937	Macrophage	38	231
Jurkat	T-lymphocyte	6	18
SKW 6.4	B-lymphocyte	6	19
CESS	"	<10	>10
Controls			
T47D (negative)	Human Breast Carcinoma	~3	~3
THP1 (positive)	Macrophage	8	190

Analysis of intact human PBMC indicated that these cells express mRNA for a "κ-like" R (5). Dual color flow cytometric analysis was utilized to assay for the binding of anti-KR(33-52) to normal human macrophage (CD14+) and T lymphocytes (CD3+). It was observed that both macrphage and T lymphocytes bound anti-κR(33-52) antibody. Anti-κR(33-52) and RGG were assessed for binding to CD14+ PBMC. The results indicate that anti-κR(33-52) bound CD14+ cells with a 15-fold increase compared to normal RGG (MFI=320 vs. MFI=21). Anti-κR(33-52) was also found to bind CD3+ cells (MFI=19 vs. RGG MFI=3) albeit less than CD14+ cells. These results indicate that anti-κR(33-52) binds normal PBMC-derived mononuclear cells as well as mononuclear cell lines, which express κR-specific mRNA.

ix. N utralization of U50,488H-mediat d suppression of lymphocyte pr lif ration by anti-κR(32-52) antibody in vitro. The results of published studies have shown that

opioid peptide-induced regulation of *in vitro* immune responses can occur via specific receptor-ligand interactions. More specifically, it has been shown that the κR-selective agonist U50,488H is capable of suppressing SAC-induced lymphocyte proliferation by human PBMC cultures (6). The inhibition of lymphocyte activation by U50,488H has also been shown to be reversed by the κR-selective antagonist nor-BNI. To determine whether anti-κR(33-52) was capable of acting as an κR selective antagonist and neutralizing U50,488H-mediated suppression, PBMC cultures were preincubated with various concentrations of protein A purified anti-κR(32-52) prior the addition of SAC and U50,488H. U50,488H suppresses SAC-induced lymphocyte proliferation in a dose dependent fashion (5). Maximal suppression was obtained when U50,488H was used at a concentration of 10^{-6} M. PBMC cultures were preincubated with various concentrations of anti-κR(33-52) (1-50 μg/ml), followed by the addition of U50,488H plus SAC, and proliferation measured on day 3 of culture. Anti-κR(33-55) was found to neutralize U50,488H-mediated suppression of SAC-induced lymphocyte proliferation in a dose dependent fashion. In contrast, identical concentrations of normal RGG failed to inhibit κR selective agonist mediated immunosuppression.

Since SAC has been shown to induce both T and B lymphocyte proliferation, similar experiments were conducted with the T cell mitogen PHA. Anti-KR(33-52) was also able to neutralize the ability of U50,488H to suppress mitogen-induced T cell proliferation. U50,488H (10^{-6} M) suppressed PHA-induced T cell proliferation by 85%. This suppression was reversed by preincubating the cells with anti-κR(33-52). Preincubation of PBMC with normal RGG failed to block U50,488H -mediated suppression of T cell proliferation.

Anti-κR(33-52) does not appear to directly modulate lymphocyte prolif ration. The co-culture of PBMC with anti-κR(33-52), in the absence of mitogen, failed to

stimulate the cells above the media control. Moreover, the combination of antiκR(33-52) and PHA or SAC did not result in increased cell proliferation compared to PBMC cultures receiving mitogen only.

x. Neutralization of U50,488N-mediated suppression of IgG synthesis by anti-κR(32-52) antibody in vitro. In addition to lymphocyte proliferation, U50,488H is a potent inhibitor of SAC-induced IgG synthesis in human PBMC cultures (6). To determine whether anti-KR(32-52) was capable of neutralizing the suppression of IgG synthesis, PBMC were preincubated with anti-κR(32-52) followed by the addition of U50,488H and SAC, and IgG levels measured on day 10. Results indicate that U50,488H at 10^{-8} M and 10^{-7} M inhibited IgG synthesis by 67% and 85% respectively (5). The inclusion of anti-κR(32-52) in culture was found to neutralize suppression of SAC induced IgG synthesis in a dose dependent manner. In contrast, similar concentrations of normal RGG failed to neutralize the observed suppression.

To assess the specificity of anti-κR(32-52) antibody, PBMC were incubated with specific antibody or RGG followed by co-culture with U50,488H or the μ receptor selective agonist (DAGO) and IgG production measured by ELISA. The results indicate that, whereas, anti-κR(32-52) neutralized U50,488H-mediated inhibition of SAC-induced IgG synthesis, anti-κR(32-52) was unable to neutralize DAGO-mediated suppression of IgG synthesis.

These results indicate that in addition to binding lymphocytes and macrophage, anti-κR(32-52) is capable of neutralizing the ability of a κR selective agonist (U50,488H), but not a μR selective agonist (DAGO). Additionally the antibody demonstrated significant inhibition of both lymphocyte proliferation and differentiation to antibody synthesis. These results further demonstrate the specificity of anti-κR(33-52) for

the human kappa receptor.

As can be seen from the antibody binding data presented above, the site directed polycolonal antibodies raised in rabbits using the C5a-agonist form of the molecular adjuvant conjugated to the κ receptor sequence were capable of binding to normal human cells and cell lines expressing mRNA specific for the human κ receptor. Flow cytometric analysis of a neuronal cell line (NT2), normal blood-derived CD14+ monocytes, monocyte-like cell ines (U937 and THP1), normal blood derived CD3+ T cells and a T cell line (Jurkat), and human B cell lines (SKW6.4 and CESS) revealed that the cells were all bound by anti-κR(33-52) in a specific manner. The anti-κR(33-52) did not bind to a cell line determined not to express mRNA for the human κ receptor.

Anti-κR(32-52) was found to specifically neutralize κR-selective agonist (U50,488H)-mediated inhibition of lymphocyte activation. The antiserum was found to neutralize, in a dose dependent manner, U50,488H-mediated inhibition of: 1) SAC-induced lymphocyte proliferation; 2) PHA-induced lymphocyte proliferation and; 3) SAC-induced IgG synthesis. In contrast, DAGO-mediated suppression of SAC-induced IgG production was not affected by anti-κR(32-52). These results suggest that this site directed polyclonal antiserum specifically interacts with the human κR on PBMC. The results presented indicate that polyclonal anti-κR(32-52) antibodies interact with the exposed N-terminal region of the κR. While this antiserum effectively blocked U50,488H -mediated lymphocyte activation, it did not activate macrophage or lymphocytes.

While anti-κ opioid receceptor antibodies are exemplified above, conjugation of C5a agonist peptide to peptides corresponding to μ and Δ specific peptides has resulted in the successful generation of specific antibodies to the μ and Δ epitopes.

EXAMPLE 4.

Comparison of Immunogenicity of Epitope-C5a agonist constructs with epitope-KLH conjugates.

The following experiment was performed in order to compare the potency of the molecular adjuvant of the present invention with a widely used method for enhancing the immune response to peptide epitopes. The objective was a direct comparison of the response to a construct of MUC1 epitope-C5a agonist and the same epitope conjugated to keyhole limpet hemocyanine (KLH) in mice. The results are summarized in Table 2.

Table 2

MUC1 Specific Ab Isotype Titers Produced with Different Immunogens.

Ab Isotypes and Titers[a]

	IgA	IgG1	IgG2a	IgG2b	IgG3	IgM
YKQGGFLGLYSFKPMPLaR[b]	0	0	1260 (5/5)	1780 (5/5)	0	6310 (5/5)
YKQGGFLGL-KLH[c]	0	100 (2/5)	0	0	0	5010 (4/5)

[a] Sera were screened against MUC1 peptide and mean titer values of responders are shown. Parentheses indicate the number of responders. Ab titer is defined as the sera dilution within the linear range at which specific reactivity is lost.

[b] Five C57BL6 mice were immunized and boosted with YKQGGFLGLYSFKPMPLaR and sera were obtained as indicated in the Material and Methods section. Standard error of responder titer values was less than 32% for all isotypes.

[c] Five C57BL6 mice were immunized and boosted with YKQGGFLGL-KLH and sera were obtained as indicated in the Materials and Methods section. Standard error of responder titer values was less then 25% for IgM and less than 40% for IgG1.

A similar experiment was performed in rabits. The immunogens used in rabbits were the κ- and μ- opioid

receptor epitopes, FPGWAEPDSNGSEDAQL and GDLSDPCGNRTNLGGRDSL, respectively. The serum antibody titer and antibody subtypes produced in rabbits injected with the two compositions containing the different immunogens were compared.

i. Peptide conjugates. In one instance the epitopes were conjugated to KLH via a lysine residue added synthetically to the N-terminus of the epitope along with an alanine residue which acted as a spacer. In this experiment, glutaldehyde was used to effect conjugation. In the another case, the epitopes were linked to the N-terminal end of the C5a agonist YSFKPMPLaR using the solid phase peptide synthetic methodologies described above in example 1.

ii. Immunization protocol for rabbits. Rabbits were immunized s.c. with 500 μg of either the epitope-KLH or the epitope-YSFKPMPLaR constructs in compete Freund's adjuvant (GIBCO, Grand Island, NY). Booster injections were administered on days 30 and 60 in incomplete Freund's adjuvant. Serum was collected starting at day 60 post-immunization.

iii. Antibody determination. The presence of rabbit IgG specific for the peptide epitopes was determined by ELISA as previously described (8).

Rabbits immunized with the epitope-C5a agonist generated high titer IgG Abs specific for the opioid receptor peptide epitopes. The rabbits immunized with the opioid receptor epitopes conjugated to the carrier protein KLH also produced high titer antibodies specific epitopes to which they were injected. These results demonstrate that the decapeptide C5a-agonist was as effective as the large molecular weight protein, KLH, conjugated to the epitope at inducing specific anti-peptide antibodies in non-rodent species.

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While certain preferred embodiments of the present invention have been described and specifically exemplified abov , it is not intended that the invention be limited to such embodiments. Various modifications

WHAT IS CLAIMED IS:

1. A molecular adjuvant for enhancing an immune response to an immunogen comprising:
a targeting ligand having binding affinity for a characteristic determinant of an antigen presenting cell, said targeting ligand being functionally linked to said immunogen, whereby binding of said molecular adjuvant to said antigen presenting cell determinant activates said antigen presenting cell, effecting delivery of said immunogen to an antigen presenting pathway of said antigen presenting cell.

2. A molecular adjuvant as claimed in claim 1, wherein said targeting ligand binds specifically to a determinant comprising an immunomodulatory receptor of said antigen presenting cell.

3. A molecular adjuvant as claimed in claim 2, wherein said targeting ligand binds specifically to a receptor selected from the group consisting of C5a receptor, IFN-gamma receptor, CD21 (C3d) receptor, CD64 (FcgRI) receptor, and CD23 (FceRII) receptor.

4. A molecular adjuvant as claimed in claim 3, wherein said targeting ligand binds specifically to a C5a receptor and is selected from the group consisting of C5a and a peptide agonist analog of C5a comprising the C-terminal ten residues of C5a.

5. A molecular adjuvant as claimed in claim 4, wherein said targeting ligand is a peptide comprising the sequence YSFKPMPLaR, which is Sequence I.D. No. 1.

6. A molecular adjuvant as claimed in claim 1, comprising a targeting ligand and an immunogen

13. A molecular adjuvant as claimed in claim 10, wher in said immunog n comprises a protein.

14. A molecular adjuvant as claimed in claim 13, wherein said protein comprises serum amyloid A (SAA).

15. A molecular adjuvant as claimed in claim 14 having the formula SAA-K-Ahx-YSFKPMPLaR.

16. A molecular adjuvant as claimed in claim 1, wherein said immunogen comprises a tumor-specific antigen.

17. A composition for enhancing an immune response to an immunogen in a subject in which said enhanced immune response is desired, said composition comprising the molecular adjuvant of claim 1 in a biologically compatible medium.

18. A method for activating an antigen presenting cell for inducing an enhanced immune response to an immunogen, said immunogen being delivered to the antigen presenting pathway of said antigen presenting cell, said method comprising binding to a characteristic surface determinant of said antigen presenting cell a molecular adjuvant as claimed in claim 1.

19. A method as claimed in claim 18, wherein binding of said molecular adjuvant to said antigen presenting cell induces a humoral immune response.

20. A method as claimed in claim 18, wh rein binding of said molecular adjuvant to said antigen presenting cell induc s a cellular immune

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rul 70)

Applicant's or agent's file reference UNMC.63102	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US96/16825	International filing date *(day/month/year)* 18 OCTOBER 1996	Priority date *(day/month/year)* 20 OCTOBER 1995
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant UNIVERSITY OF NEBRASKA BOARD OF REGENTS		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 27 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [X] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20 MAY 1997	15 NOVEMBER 1997
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer F. PIERRE VANDERVEGT
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US96/16825

I. Basis f the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

☐ the international application as originally filed.

☒ the description, pages (See Attached) , as originally filed.
pages _____ , filed with the demand.
pages _____ , filed with the letter of _____.
pages _____ , filed with the letter of _____.

☒ the claims, Nos. (See Attached) , as originally filed.
Nos. _____ , as amended under Article 19.
Nos. _____ , filed with the demand.
Nos. _____ , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

☒ the drawings, sheets/~~fig~~ (See Attached) , as originally filed.
sheets/~~fig~~ _____ , filed with the demand.
sheets/~~fig~~ _____ , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/~~fig~~ NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US96/16825

V. Reasoned statement under Article 35(2) with regard t n velty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-24	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-24	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-24	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS -

Claims 1-24 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest immunogens linked molecular adjuvants which incorporate a targeting ligand having binding affinity for a determinant of an antigen presenting cell.

The Dempsey et al. reference cited on the International Search report teaches a molecular adjuvant comprising a portion of the C3 component of complement which binds to CD21 on the surface of B cells. However, the Dempsey et al. reference was published after the 20 October 1995 priority date for this application.

The Suntory Kabushiki Kaisha reference is a state of the art reference which teaches a peptide which comprises SEQ ID NO: 4, a segment of human interferon gamma and describes it as a physiologically active polypeptide. However, the reference does not teach or suggest its use as a molecular adjuvant.

The Sanderson et al. reference is a state of the art reference which teaches decapeptide analogs of the C-terminal region of C5a anaphylatoxin which bind to the C5a receptor on cells and serve as agonists of neutrophil activity. However, the reference does not teach the linking of an antigenic peptide to the molecule(s) for use as a molecular adjuvant.

---------- NEW CITATIONS ----------

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US96/16825

VIII. Certain observati ns on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 6 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: The claim recites a specific amino acid sequence but does not identify its SEQ ID NO.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII Sheet 10

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): A61K 38/08, 38/10, 38/17, 38/21, 38/30, 39/385, 39/39; C07K 1/04, 7/06, 7/08, 14/52, 14/705, 14/74, 17/00 and
US Cl.: 424/185.1,192.1,277.1;530/324,326,328,395,403

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
pages, 1-7, 9-10, 12-17, 20, 22-24, 27, 32-34, 42, 50, as originally filed.
pages, NONE, filed with the demand.
and additional amendments:
Pages 8, 11, 18, 19, 21, 25, 26, 28-31, 35-41, 43-49 filed with the letter of 10 October 1997.

This report has been drawn on the basis of the claims,
numbers, 7-12, 21-24, as originally filed.
numbers, NONE, as amended under Article 19.
numbers, NONE, filed with the demand.
and additional amendments:
Claim 1-6, 13-20 filed with the letter of 10 October 1997.

This report has been drawn on the basis of the drawings,
sheets, 1-4, as originally filed.
sheets, NONE, filed with the demand.
and additional amendments:
NONE

WO 97/14426
PCT/US96/16825

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

HAGAN, Patrick, J.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philadelphia, PA 19103
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 24 April 1997 (24.04.97)	
Applicant's or agent's file reference UNMC.63102	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US96/16825	18 October 1996 (18.10.96)	20 October 1995 (20.10.95)

Applicant
UNIVERSITY OF NEBRASKA BOARD OF REGENTS et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CA,EP,JP,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   None

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   24 April 1997 (24.04.97) under No. WO 97/14426

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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